PATENT
Docket No. 381092000700

CERTIFICATE OF MAILING BY "FIRST CLASS MAIL

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on June 4, 2001.

Jami M Procopio

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#36
M.Q
3/26/03

In the application of:

Terry P. Snutch, *et al.*

Serial No.: 09/030,482

Filing Date: 25 February 1998

For: NOVEL HUMAN CALCIUM CHANNELS AND RELATED PROBES, CELL LINES AND METHODS

Examiner: Nirmal S. Basi

Group Art Unit: 1646

EXPEDITED PROCEDURE -- EXAMINING GROUP 1646

DECLARATION OF DR. TERRANCE SNUTCH

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Terrance Snutch, declare as follows:

1. I am a co-inventor of the subject matter claimed in the above-referenced application and have been practicing in the field of molecular biology, and specifically in the field of ion channels, for over 15 years. A copy of my *curriculum vitae* is attached hereto as Exhibit A. I have published many papers on the structure and function of calcium channels and am considered one of the leading researchers in this field.

2. The nucleotide sequence set forth as SEQ. ID. NO: 18 encodes about 85% of the total amino acid sequence, starting at the exact N-terminus, of a member of a family of voltage-gated ion channels that contain four homologous structural domains (domains I, II, III and IV). All of the identified members of this four-domain class of ions channels are either voltage-gated

Exhibit A

calcium or sodium ion channels. In each case, each of the four homologous domains contains structural elements necessary for channel function. Each contains six transmembrane segments including a transmembrane segment called the S4 region that acts as the voltage sensor of the channel. Also contained in each homologous domain is a region called the P-loop or Pore region that contains specific amino acid residues responsible for ion selectivity. Voltage-gated sodium and calcium channels can easily be distinguished from each other based upon their overall degree of sequence conservation and by the specific amino acid residues that constitute the Pore region responsible for ion flux (see below).

3. I have concluded that SEQ. ID. NO: 18 encodes virtually the entire amino acid sequence of a major branch of the calcium channel family that represents a T-type channel. The calcium channel family and the evolutionary relationships between its members are shown in Figure 1. The construction of the family is based on sequence homology in the genes encoding the various members. As seen in Figure 1, a closely related branch of the family is represented by several types designated P/Q, N and R; this major branch is more distantly related to another branch which is represented by various L-type channels. The nucleotide sequence set forth in the present invention as SEQ. ID. NO: 18 has characteristics which place it in this general family, but it is relatively distantly related to the two branches represented by the α_1 subunits A, B, E and α_1 subunits S, C and D. My conclusion that Figure 1 represents an accurate characterization of SEQ. ID. NO: 18 is based on the reasoning set forth in paragraphs 4-6 below.

4. Over the past 30 years native calcium channels have been classified into high-threshold (L-type, N-type, P/Q-type and R-type) or low threshold subtypes (T-type), as illustrated in Figure 1. The placement of the high-voltage types is based on evolutionary analysis of already cloned calcium channels. This shows that the P/Q-type, N-type and R-type calcium channel α_1 subunits constitute one branch of calcium channels while the L-type subunits α_1C, α_1D and α_1S constitute a second evolutionary branch. The only class of calcium channel not accounted for is the third branch which would thus include the T-type. Comparison of SEQ. ID. NO: 18 deduced amino acid with that of the other α_1 subunits clearly indicates that it forms a third evolutionary class of calcium channel, which based upon physiological and pharmacological criteria must represent the T-type channel.

5. Examination of the Pore region of SEQ. ID. NO: 18 compared to the high threshold calcium channels and also sodium channels indicates that SEQ. ID. NO: 18 encodes a novel type

of calcium channel. In all high threshold calcium channels, the pore region of each domain (I, II, III, and IV) contains a glutamate residue (E) that is responsible for the selective flux of calcium through the channel (Yang, *et al.*, 1993, "Molecular Determinants of Ca Selectivity and Ion Permeation in L-type Ca Channels," *Nature* 366:158-161). In contrast, sodium channels possess other amino acids in the analogous positions (domain I = aspartate (D), domain II = glutamate (E), domain III = lysine (K). Mutation of the domain III lysine (K) of sodium channels to the corresponding glutamate (E) found in the high threshold calcium channels results in the flux of calcium and indicates that the domain III pore region is critical to defining ion flux (Heinemann, *et al.*, 1992, "Calcium Channel Characteristics Conferred on the Sodium Channel by Single Mutations," *Nature* 356:441-443). It is known from the behavior of the calcium channels whose genes had not yet been cloned that these "T-type" calcium channels possess distinct permeation properties compared to high-threshold calcium channels. In general, while high-threshold calcium channels flux barium at a higher rate than calcium, T-type channels flux calcium at a similar or higher rate than that for barium (for review, see Huguenard, 1996, "Low-Threshold Calcium Currents in the Central Nervous System Neurons" *Annu. Rev. Physiol.* 58:329-348). Examination of the Pore region of SEQ. ID. NO: 18 shows that in domain III it contains the substitution of an aspartate residue (D) for the glutamate residue (E) that is absolutely conserved in all of the high threshold calcium channels. A comparison of the relevant positions in the Pore regions of domains I, II and III of the known calcium channels, the sodium channel, and SEQ. ID. NO: 18 is shown in Figure 2. Since the domain III glutamate (E) residue of the high threshold calcium channels is critical for ion flux (Yang, *et al.*, 1993, *supra*), one can conclude that the substitution of D for E in SEQ. ID. NO: 18 contributes to the unique permeation properties of the T-type channel.

6. The intracellular linker region separating domains I and II of all high threshold calcium channels contains a high-affinity binding site for the calcium channel β subunit. The consensus β subunit binding site found in the N-type, P/Q-type, L-type and R-type channels is: QQ-E—L-GY—WI---E and is a defining characteristic of high threshold calcium channels (Pragnell, *et al.*, 1994 "Calcium Channel β-Subunit Binds to a Conserved Motif in the I-II Cytoplasmic Linker of the α_1 Subunit," *Nature* 368:67-70). It is known that T-type calcium channels do not contain or bind to the β subunit of high threshold calcium channels. The amino

acid sequence encoded by SEQ. ID. NO: 18 does not possess the consensus β subunit binding sequence. This is consistent with SEQ. ID. NO: 18 encoding a T-type calcium channel.

7. For the reasons set forth in paragraphs 4-6, I am certain that SEQ. ID. NO: 18 encodes a T-type calcium ion channel α_1 subunit. To summarize, as set forth in paragraph 4, a comparison of the deduced amino acid sequence in SEQ. ID. NO: 18 with that of other known four-domain voltage gated channels shows that it is relatively distantly related from the calcium channels whose genes have already been cloned and amino acid sequences deduced and thus belongs to the low-threshold subtype (T-type) which had not heretofore been cloned; paragraph 5 demonstrates that the critical amino acids in the Pore region verify that it is a calcium ion channel rather than a sodium ion channel and that it is distinct from the already cloned high-threshold channels, and paragraph 6 demonstrates that as expected, the deduced amino acid sequence lacks a β subunit binding sequence. The coding sequence is not entirely complete (approximately 85%), but it is sufficient both to (1) verify its nature as encoding a T-type calcium ion channel subunit and (2) to provide sufficient information to permit supplementation with additional nucleotide sequence to encode a functional T-type channel without retrieving a full length clone.

8. SEQ. ID. NO: 18 contains an ATG start codon that precedes three complete homologous structural domains I, II and III, and up to the S1 transmembrane segment of domain IV. Each of the domains I through III possesses an intact voltage sensor segment (S4) as well as a complete Pore region. Since structural domains II, III and IV result from the evolutionary duplication of domain I, SEQ. ID. NO: 18 contains sufficient information to construct a complete domain IV and thus a functional T-type calcium channel α_1 subunit. The nature of the amino acid sequence in domain IV can be surmised from the amino acid sequence of domain III. Of course, means to construct a nucleotide sequence extending the nucleotide sequence of SEQ. ID. NO: 18 to include a nucleotide sequence encoding this deduced amino acid sequence are routine.

9. In summary, one of ordinary skill in the art given the information set forth in SEQ. ID. NO: 18

(a) would understand that it encodes about 85% of a functional T-type calcium channel α_1 subunit starting at the N-terminus,

(b) would be able to design an amino acid sequence representing the missing C-terminal portion based on homology to the three domains encoded by SEQ. ID. NO: 18, and

(c) would be able to construct an expression system containing a nucleotide sequence encoding a functional T-type calcium ion channel α_1 subunit without obtaining a full length clone.

10. In addition to the foregoing demonstration that the application discloses the essential features of a T-type calcium channel, I further provide information regarding the nexus between all T-type calcium channels and identified conditions which can be treated with compounds that interact with T-type calcium channels. There are several T-type calcium channels found in a single individual which vary slightly in structure and demonstrably in terms of their distribution among various tissues. The particular T-type calcium channel involved in a particular condition may depend on its tissue distribution; for instance, T-type channels found in the neuronal system are associated with epilepsy and neurological diseases in general where spastic convulsions are involved. However, it is not necessary to understand which particular T-type calcium channel is being used in a screen for compounds that would be useful in treating, for example, these convulsive conditions because of the similarity in the binding specificity of all T-type channels. In very simple terms, compounds which are found to inhibit the activity of neuronal T-type channels will also inhibit the activity of T-type channels found in other tissues. Thus, any arbitrarily chosen T-type channel could be expressed in a cell line for use in screening assays to identify antagonists and the antagonists would be useful in treating the conditions associated with any T-type channel. As noted in the accompanying response, abnormal T-type activity is associated with a number of cardiac conditions, with hypertension, with neurological diseases involving spastic convulsions, and with impaired fertility. An antagonist identified with regard to any T-type channel would be useful in all of these conditions.

11. The pattern of similar binding activity among all T-type channels can be analogized to such a pattern among L-type channels. All of the T-type channels have similar behaviors in that they activate at low membrane potential, have small single channel conductance, have negative steady state inactivation properties, and contribute to spike firing patterns and rhythmic bursting processes. Analogous to the T-type channel another type of channel linked by similar behaviors is the L-type. There are several α_1 subunits associated with various L-type channels - *i.e.*, α_{1S}, α_{1C}, and α_{1D} and each is encoded by a distinct gene and exhibits a distinct distribution

pattern. For example, α_{1S} is in skeletal muscle; α_{1C} is in neurons and cardiac and smooth muscle; and α_{1D} is found in neurons and endocrine cells. They can be discriminated from all other types of calcium channels by their common sensitivity to 1,4-dihydropyridines. Thus, any one of these genes could be used to generate an L-type calcium channel for use in a cell-based assay to identify antagonists. These identified antagonists would bind to all of these L-type channels and thus would be useful in treating conditions related to any one of them.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at VANCOUVER, BC on 29 May 2001.

Terrance Snutch

PATENT
Docket No. 381092000700

CERTIFICATE OF MAILING BY "FIRST CLASS MAIL"

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on October 10, 2000.

Karen LePari
Karen LePari

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Terry P. Snutch, *et al.*

Serial No.: 09/030,482

Filing Date: 25 February 1998

For: NOVEL HUMAN CALCIUM CHANNELS AND RELATED PROBES, CELL LINES AND METHODS

Examiner: Nirmal S. Basi

Group Art Unit: 1646

DECLARATION OF TERRANCE P. SNUTCH

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Terrance P. Snutch, declare as follows:

1. I am a co-inventor of the subject matter claimed in the above-referenced application and have been practicing in the field of molecular biology for over twenty years. I am very familiar with cloning techniques and hybridization conditions.

2. In my opinion, the designation of hybridization conditions as those of medium stringency would convey to those of skill in the art that nucleotide sequences encoding closely related members of the same family of molecules would hybridize, but those outside this closely related family would not. In the present case, it would be understood that members of the family

Exhibit B

represented by the newly discovered ion channel family described herein would hybridize to the disclosed nucleotide sequences, but nucleotide sequences encoding ion channels presently known in the art would not.

3. There are many examples in the literature whereby hybridization stringency is referred to as high, medium or low based upon temperature and salt conditions (*e.g.* Snutch, T.P., Heschl, M.F.P. and Baillie, D.L. (1988). The Caenorhabditis elegans hsp70 gene family: a molecular genetic characterization. *Gene* 64:241-255; Yu, A.S.L., Hebert, S.C., Brenner, B.M. and Lytton, J. (1992). Molecular characterization and nephron distribution of a family of transcripts encoding the pore-forming subunit of Ca2+ channels in the kidney. *Proc. Natl. Acad. Sci. USA* 89:10494-10498).

4. Medium stringency hybridization to colony or plaque lifts fixed on nitrocellulose or nylon membranes is typically performed 62°C to 65°C in the presence of probe in a solution containing 5 times Denhardt's, 0.3% SDS and 5 X SSPE. Non-specific carrier DNA such as denatured salmon sperm DNA (100 to 200 ug/ml) and a hybridization accelerator such as dextran sulfate (10%) may also be included in the hybridization buffer. Alternatively, medium stringency hybridization may be performed at 42°C in a solution containing 50% formamide, 5 times Denhardt's, 0.3%SDS and 5 X SSPE. The exact concentration of SDS and SSPE can vary and there are reports of SDS utilized from 0.2% to 0.7% and SSPE from 5 X to 6 X or the alternative buffer SSC from 5 X to 6 X. After hybridization the hybridization solution is removed and membranes are washed several times in a solution typically containing 0.1 % to 0.3% SDS and 2 X SSPE to 0.2 X SSPE. The temperature of medium stringency washing typically can vary from 55°C to 65°C.

5. Detailed information concerning the relevant considerations to be taken into account for determining hybridization stringency can be found in: Basic Methods in Molecular Biology (1986) Edited by Davis, L.G., Dibner, M.D. and Battery, J.F. Elsevier Science

Publishing Co., New York, and in DNA Probes (1989) Edited by Keller, G.H and Manak, M.M. Stockton Press, New York.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Executed at Vancouver, BC on 10 October 2000.

Terrance P. Snutch

Jpn. J. Pharmacol. 85, 339 – 350 (2001)

REVIEW

Molecular Pharmacology of T-type Ca^{2+} Channels

Tiffany N. Heady[1], Juan C. Gomora[2], Timothy L. Macdonald[1] and Edward Perez-Reyes[2,*]

[1]*Department of Chemistry, University of Virginia, Charlottesville, Virginia 22904, USA*
[2]*Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908, USA*

Received November 9, 2000

ABSTRACT—Over the past few years increasing attention has been focused on T-type calcium channels and their possible physiological and pathophysiological roles. Efforts toward elucidating the exact role(s) of these calcium channels have been hampered by the lack of T-type specific antagonists, resulting in the subsequent use of less selective calcium channel antagonists. In addition, the activity of these blockers often varies with cell or tissue type, as well as recording conditions. This review summarizes a variety of compounds that exhibit varying degrees of blocking activity towards T-type Ca^{2+} channels. It is designed as an aid for researchers in need of antagonists to study the biophysical and pathological nature of T-type channels, as well as a starting point for those attempting to develop potent and selective antagonists of the channel.

Keywords: T-type calcium channel, Antihypertensive, Mibefradil, Antiepileptic, Anesthetic, Antipsychotic, Amiloride

Introduction

Calcium is essential for life and is the most common signal transduction element in cells. A staggering electrochemical gradient exists between its extracellular concentration of approximately 2 mM compared to resting intracellular concentrations of approximately 100 nM. Stimulation of cells can lead to increases in intracellular concentrations into the micromolar range, with much higher concentrations in microdomains. This calcium enters the cytosol either through plasma membrane ion channels or is released from intracellular pools. Plasma membrane ion channels can be classified as either receptor- or store-operated channels, which are usually non-selective for cations such as Na^+ and Ca^{2+}, or voltage-gated channels, which are highly selective for Ca^{2+}.

The diversity of voltage-gated calcium channels has been extensively studied using electrophysiological, biochemical, pharmacological, and more recently, using molecular biology techniques. Early voltage-clamp studies identified two main classes of channels: those that responded to small (10 mV) changes in the resting membrane potential, or low-voltage-activated (LVA), and those that required stronger (30 mV) depolarizations to open, or high-voltage-activated (HVA). Pharmacological studies allowed further dissection of the HVA family into L-, N-, P-, Q-, and R-types (1). These studies relied heavily on toxins isolated from snails (*Conus*) and funnel-web spiders (*Agelenopsis*) (2). Cloning of ten $\alpha 1$ subunits of voltage-activated Ca^{2+} channels has allowed a new classification based on sequence similarities (3). Alignments reveal that there are three main subfamilies of $\alpha 1$ subunit: 1) Ca_v1, which contains four members that encode L-type channels ($Ca_v1.1$, $\alpha 1S$; $Ca_v1.2$, $\alpha 1C$; $Ca_v1.3$, $\alpha 1D$; and $Ca_v1.4$, $\alpha 1F$, which has yet to be expressed); 2) Ca_v2, which contains three members including the P- and Q-types (both encoded by $Ca_v2.1$, $\alpha 1A$), the N-type ($Ca_v2.2$, $\alpha 1B$), and R-type ($Ca_v2.3$, $\alpha 1E$); and 3) Ca_v3, which contains three members that encode T-type channels ($Ca_v3.1$, $\alpha 1G$; $Ca_v3.2$, $\alpha 1H$; and $Ca_v3.3$, $\alpha 1I$). All of these $\alpha 1$ subunits contain structural motifs that have been extremely conserved during evolution. The most basic element appears to be a pore loop surrounded by two membrane spanning domains (4). During evolution, more membrane spanning regions were added, resulting in a structure containing 6 transmembrane regions and a pore loop. One of these transmembrane regions contains many positive charges, conferring voltage sensitivity to the channel. Such is the structure of many voltage-gated K^+ channels (K_v), which are composed of four of these subunits. The next step in evolution was to join four α subunits into a single molecule, which is the structure of both voltage-gated Ca^{2+} and Na^+ channels. This conservation of sequence and predicted

*Corresponding author: FAX: +1-804-982-3878
E-mail: eperez@virginia.edu

Exhibit C

structure allows comparisons of these channels to the *Streptomyces* K^+ channel whose structure was recently solved using X-ray crystallography (4). Such comparisons may be useful in understanding drug binding sites (5).

Biochemical studies established that HVA channels contain auxiliary subunits such as α_2, β, δ, and γ. Subsequent studies showed that δ was encoded on the same gene as α_2. Molecular cloning has revealed that each of these subunits contains other family members. There are now genes for three $\alpha_2\delta$ subunits, four β subunits and five γ subunits (6, 7). Coexpression studies of the cloned subunits indicates that auxiliary subunits can alter the pharmacological properties of the channel (8). Furthermore, auxiliary subunits themselves may be the target of drugs; for example, gabapentin binds with high affinity to $\alpha_2\delta$ (9). The structure of LVA channels has not been determined biochemically.

Although the subject of this review is the pharmacology of T-type calcium channels, it should be noted that presently there are no compounds that are highly selective for these channels. Studies on the pharmacology of T-type channels have been complicated by many factors. For one, these channels are rarely expressed alone, which means the currents can be contaminated by other ionic conductances, notably HVA channels. This leads to under-estimates of the drug's potency. Typically LVA currents are separated from HVA currents by changing the holding potential from −90 mV (where both are active) to −40 mV where only some HVA channels are active. However, the block of many ion channels is state-dependent, with up to 1000-fold higher affinity for partially inactivated states. This may also lead to under-estimates of a drug's selectivity because the effects are being measured on LVA channels in the resting state, while HVA channels may be partially inactivated. Most of the studies reported herein used resting membrane potentials ranging from −100 to −80 mV, so the reported IC_{50} values are probably dominated by the affinity of the drug for the resting state. Second, T-type channels are typically expressed at low densities, so many studies have used high concentrations of charge carrier to amplify the small currents. Again, this may lead to under-estimates of potency because channel block is often reduced at high divalent cation concentrations. Although this effect is often attributed to interactions in the permeation pathway, it should be noted that surface charge screening may also play a role. Due to these considerations, the divalent cation and its concentration are noted in the tables.

Antihypertensives

Calcium channel blockers have been used since the early 1960s to treat a variety of cardiovascular diseases like hypertension, cardiac arrhythmia and angina pectoris (10). Traditional calcium channel antagonists fit into three distinct chemical classes, the phenylalkylamines (e.g., verapamil), dihydropyridines (e.g., nifedipine) and benzothiazepines (e.g., diltiazem). Representative structures are shown in Fig. 1. In general, their beneficial effects are thought to be due to blocking smooth muscle L-type channels, leading to decreases in cytosolic Ca^{2+}, and relaxation of vascular smooth muscle cells. An important property of these drugs is to block preferentially smooth muscle L-type channels with little effect on cardiac L-type channels. Many first generation calcium channel blockers blocked both, such as verapamil and diltiazem. Second generation blockers such as nimodipine display greater selectivity for vascular beds (11). Two mechanisms that contribute to this selectivity are: 1) state dependent block, where drugs preferentially block partially inactivated channels, and 2) tissue specific expression of $Ca_v1.2$ splice variants that have distinct sensitivity to the drugs (12).

Mibefradil (Ro 40-5967) was introduced to the market in 1997, but then abruptly withdrawn. It was approved for use in hypertension and angina and marketed as the first selective T-type Ca^{2+} channel blocker (13). Indeed, depending on the cell type, mibefradil blocks T-type Ca^{2+} channels 10 to 30 times more potently than L-type Ca^{2+} channels (14, 15). In addition, mibefradil is highly tissue selective, relaxing smooth muscle without inducing reflex tachycardia, or having much effect on cardiac chronotropy or inotropy (13, 16). However, pharmacokinetic interactions with other drugs metabolized by cytochromes P-450 3A4 and 2D6 (antihistamines, such as astemizole) eventually led to the withdrawal of mibefradil from the clinic.

Electrophysiological experiments in a wide variety of native T-type Ca^{2+} currents have shown mibefradil IC_{50} values ranging from 0.1 to 4.7 μM (Table 1). Vascular smooth muscle T-type currents have been the most sensitive to mibefradil with an IC_{50} of 0.1 – 0.2 μM (14). In contrast, T-type currents recorded from mouse spermatogenic cells were reported to be much less sensitive (IC_{50} = 4.7 μM (17)). Mibefradil blocks all three cloned T-type channels with similar potency (18), although $Ca_v3.3$ may be twofold less sensitive (19). Direct comparisons indicate that it blocks native and the cloned $Ca_v3.2$ channels with equal potency (20).

Mibefradil can also block HVA channels. In cultured spinal motoneurons, mibefradil blocked N, L-, and P/Q-type channels at similar concentrations (1.4 μM) as the block of an LVA current (21). In cerebellar Purkinje neurons, mibefradil blocked P-type currents with an apparent IC_{50} of 3 μM; however, under identical conditions, it blocked T-type currents with an IC_{50} of 14 nM (22). The major difference in these studies was the holding potential, which was −90 mV in the motoneuron study and −70 mV in the Purkinje neuron study. Although this discrepancy could be caused by differences in voltage-dependent block (discussed below), this does not appear to be the case (23).

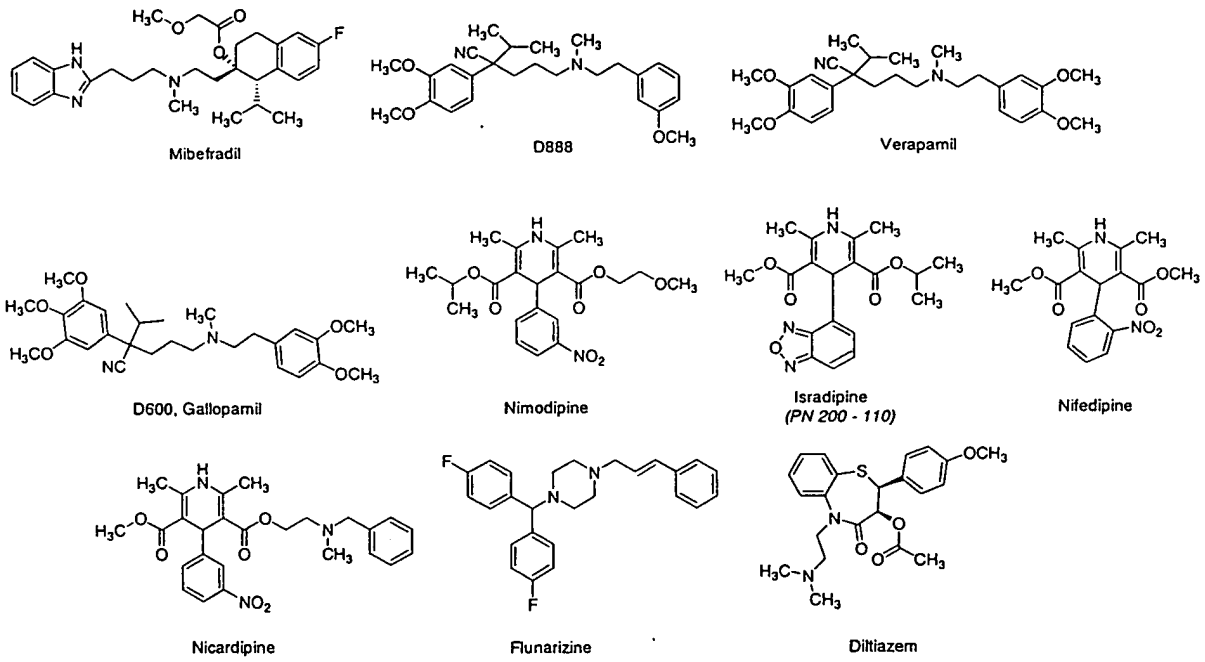

Fig. 1. Calcium channel blockers that also inhibit T-type channels.

Table 1. Calcium channel blockers that also inhibit T-type channels

	Mibefradil	Verapamil	D600	Amlodipine	Nimodipine	Isradipine	Nifedipine	Nicardipine	Flunarizine	Diltiazem	Reference
Hypothalamus			50		7		5	3.5	0.7	70	(32)
Amygdala neurons			65					2.5	1.1	120	(84)
CA1 hippocampus			120					1.6	1.2	210	(85)
Cerebellar Purkinje	1										(22)
Thalamus LD							2.6				(33)
Retinal ganglion							10 (50%)				(86)
Motoneuron	1.4						10 (approx. 5%)				(21)
Sensory neuron	3					5 (11%)	5 (19%)	2			(38, 50)
NG108-15	1 (50%)				10 (63%)						(87)
NIE-115							30 (NE)		30 (40%)		(88)
Atrial myocytes	1.2			5.7							(20)
Ventricular myocytes									10 (80%)		(89)
Aorta SMC		30						0.6	0.1	30	(39)
Azygos vein SMC	approx. 0.1										(14)
Adrenal fasciculata	1.0										(27)
Spermatocytes	5	70				0.04	0.4				(61)
Pituitary GH3			51				50			131	(90)
Thyroid hMTC	2.7	10				4.7	10 (NE)				(29)
Skeletal muscle	0.7										(25)
Ca,3.1	0.4 – 1.2										(18, 19, 30)
Ca,3.2	1.1 – 1.2	1 (17%)		31	10 (44%)						(18, 20, 67)
Ca,3.3	1.5 – 2.3										(18, 19)

All concentrations reported are micromolar and represent the IC_{50} values determined from dose-response measurements. Studies where only a single concentration was studied are noted by giving the concentration and the percent inhibition observed or NE if no significant effect was observed. The charge carrier used in each study is as follows (in mM): (reference 32) 5 Ca^{2+}, (84) 10 Ca^{2+}, (85) 10 Ca^{2+}, (22) 5 Ba^{2+}, (33) 2 Ca^{2+}, (86) 10 Ca^{2+}, (21) 5 Ba^{2+}, (38) 5 Ba^{2+}, (50) 10 Ba^{2+}, (87) 10 Ba^{2+}, (88) 20 Ba^{2+}, (20) 1.8 Ca^{2+}, (89) 5.4 Ca^{2+}, (39) 20 Ca^{2+}, (14) 20 Ba^{2+}, (27) 10 Ca^{2+}, (61) 10 Ca^{2+}, (90) 10 Ca^{2+}, (29) 30 Ba^{2+}, (25) 10 Ba^{2+}, (18) 10 Ba^{2+}, (19) 2 Ca^{2+}, (30) 20 Ba^{2+}, (67) 15 Ba^{2+}.

Many studies indicate that mibefradil is also capable of blocking various other ion channels at micromolar concentrations. It can also block Ca^{2+}- and volume activated Cl^- channels of calf endothelial cells with IC_{50}s of approximately 5 μM, while in the same preparation, the inwardly rectifying K^+ channel was not affected at concentrations up to 30 μM (24). In skeletal myoblasts, mibefradil blocked three voltage-gated K^+ currents, including an inward rectifier, a delayed rectifier, and a human *ether-a-go-go* with a respective IC_{50} of 5.6, 0.3 and 0.7 μM (25). The observation that mibefradil also blocks outward K^+ currents with similar potency as T-type currents was corroborated in adrenal zona fasciculata cells, where the ATP-activated K^+ current expressed was inhibited by mibefradil with an IC_{50} of 0.5 μM (26), a concentration twofold lower than that required to inhibit T-type Ca^{2+} currents under similar conditions (27). The effect of mibefradil on K^+ currents suggest that, in the case of cardiac cells, the action potential duration, resting membrane potential or even rhythmic activity could be altered by this drug. As a result, ventricular arrhythmias may occur, and this could explain in part the cardiovascular toxicity of this drug when used in combination with antihistamines. Finally, binding experiments showed that nanomolar concentrations of mibefradil can compete with [^{3}H]-WIN17317-3 binding to Na^+ channels of guinea pig synaptic membranes; this observation was confirmed with electrophysiological experiments using GH3 cells, whose Na^+ currents were inhibited by mibefradil with an IC_{50} around 1 μM (28).

Interestingly, the apparent potency of mibefradil depends on both the charge carrier used to record the currents and its concentration. Its sensitivity is lower in Ba^{2+} than in Ca^{2+} (29) and lower at higher divalent concentrations. For example, its potency decreases 4.5- to 9-fold by increasing the charge carrier concentration from 2 to 10 mM (18). These results suggest that mibefradil could be competing with permeant ions for a binding site in the channel. These observations are particularly relevant since most studies of mibefradil's block have used 10 mM charge carrier and thus have underestimated its potency (Table 1).

Studies on the mechanism of mibefradil block of T-type currents have yielded variable results. Early studies using a thyroid cancer cell line (hMTC, or TT cells) found little evidence for state-dependent block (29). Recent studies using rat cerebellar Purkinje neurons (22), pig atrial myocytes (20) or bovine adrenocortical cells (27) have conclusively demonstrated that mibefradil clearly shows higher affinity (5- to 20-fold) for partially inactivated states of the channel. This result has been confirmed using the cloned α1G and α1H channels (18, 30). Mibefradil was found to be fivefold more potent at potentials where half the channels are inactivated (18). A possible reason this effect was not observed in hMTC cells was that the measured currents were contaminated by other Ca^{2+} channels (29). To allow comparisons between studies, the values reported in Table 1 represent block of channels measured in supra-physiological concentrations of charge carrier at well-hyperpolarized potentials. It should be noted that at physiological concentrations of Ca^{2+} and membrane potentials, mibefradil blocks α1H with an apparent IC_{50} of 70 nM (18), a value that is well within the range of clinically relevant concentrations. Since mibefradil also blocks L-type channels in a voltage-sensitive manner, it is difficult to discern whether T-type channel block is relevant to its anti-hypertensive effect.

Interestingly, the corollary has also been suggested: a block of T-type channels might contribute to the anti-hypertensive activity of dihydropyridines such as felodipine and to the antiarrhythmic activity of amiodarone and bepridil (31). Akaike and coworkers have extensively studied the sensitivity of neuronal T-type currents to calcium channel blockers (Table 1). The diphenylpiperazine flunarizine was the most potent. In fact, in hypothalamic neurons, they found that flunarizine was *more* potent at blocking T- than L-type currents, while nimodipine and diltiazem were equipotent (32). These studies demonstrated that many dihydropyridines blocked neuronal T-type currents at micromolar concentrations, with nicardipine being the most potent. Since early studies using 0.1 – 1 μM dihydropyridines had shown selective block of L-type channels with little block of T-type channels, these authors concluded that there was a sub-set of dihydropyridine-sensitive T-type channels. This hypothesis was supported in studies on the changes that occur in thalamic T-type currents during development (33). Similarly, felodipine displayed an apparent affinity for inactivated T-type channels of atrial myocytes of 13 nM, but only 700 nM for those of pituitary GH_3 cells (31).

Clearly the dihydropyridine structure-activity relationships of T- and L-type channel are different. This is most clearly demonstrated by the stereoisomers of Bay K8644: T-type channels are weakly blocked by micromolar concentrations of both (34), while for L-type channels, submicromolar concentrations of the (+) isomer effectively block the current, while the (–) isomer is an *agonist*, similar to the racemic mixture (35). Stimulation of LVA currents by Bay K8644 are very difficult to interpret directly because L-type channel gating is shifted to more negative potentials. In contrast, the stereoselectivity and potency of niguldipine was similar for both L- and T-type channels; the racemic mixture blocked T-type currents in guinea pig atrial myocytes with an IC_{50} of 0.18 μM (36).

Although the binding site for dihydropyridines is different between L- and T-type channels, the mechanism of block appears similar. Dihydropyridines appear to stabilize inactivated states of both channels, causing a negative shift in the steady-state availability curve (31, 37 – 39).

Flunarizine also blocks T-type channels in a voltage-dependent manner, while diltiazem and verapamil do not (39).

Antiepileptics

Epilepsy is a disorder of the nervous system characterized by neuronal hyperexcitability. There are many forms of epilepsy, and they have been broadly classified into either partial or generalized seizures. The drugs used to treat the symptoms of these disorders differ. Antiseizure drugs are thought to tip the balance between neuronal excitation and inhibition by two mechanisms: 1) by blocking Na^+ or Ca^{2+} channels, and hence limiting the sustained repetitive firing of neurons, or 2) by enhancing the activity of inhibitory neurotransmitters, notably gamma-aminobutyric acid (GABA). Despite the fact that few drugs have been systematically tested on both Na^+ and Ca^{2+} channels, a dogma has arisen that Na^+ channel blockers are useful in partial seizures, while T-type channel blockers are only useful in generalized seizures.

Table 2 provides numerous examples of blocking T-type channels by "Na^+ channel blockers." Perhaps the best studied is phenytoin, which clearly blocks both channels at therapeutic concentrations (40). The mechanisms of block are also similar, with higher affinity for inactivated states of the channel. Studies using cloned T-type channels indicated that $Ca_v3.2$ was the most sensitive, although the block was variable even in a cloned cell, suggesting that additional factors may be important for block (41).

Ethosuximide is considered the prototypical absence seizure drug that works by inhibition of T-type channels. This hypothesis was based on the finding that ethosuximide could partially block T-type currents in thalamic neurons at therapeutically relevant concentrations (42). Support for this hypothesis came from studies with related analogs (43). For one, T-type current block was also observed at therapeutically relevant concentrations of methyl-phenyl-succinimide (MPS), the active metabolite of methsuximide. MPS differs from ethosuximide by having a phenyl substituent at the 4 position rather than an ethyl group (Fig. 2). Second, no block was observed using the convulsant analog, tetra-methyl-succinimide. The sensitivity of T-type channels to MPS has been confirmed in many other

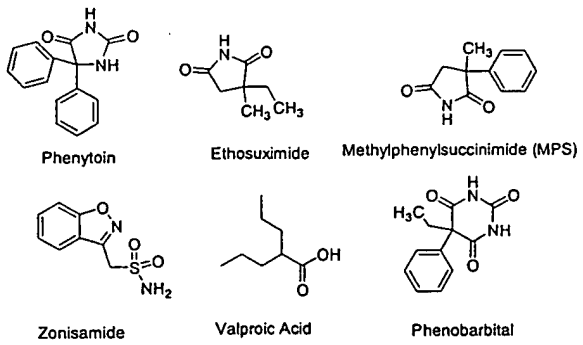

Fig. 2. Antiepileptics shown to block T-type calcium current.

Table 2. Antiepileptics shown to block T-type calcium currents

	Phenytoin	Ethosuximide	MPS	Zonisamide	Valproate	Phenobarbital	Reference
	(μM)	(mM)	(mM)	(μM)	(mM)	(mM)	
DRG	8 (max 58%)	24	0.14 (max 42%)		0.3 (max 17%)	1.7	(41, 50)
DRG		0.007					(44)
Nodose ganglion					1 (16%)		(91)
NB1	30 (50%)			50 (38%)			(92)
Cerebral cortex				500 (60%)			(93)
Thalamus VB	100 (33%)	0.2 (max 40%)	1.1		1 (NE)		(42)
Thalamus VB			3 (52%)				(94)
Thalamus nRT			3 (53%)				(94)
Hippocampus CA1	100 (43%)						(57)
GH3	100 (36%)	2.5 (10%)					(90)
$Ca_v3.1$	140	14	1.7		3 (4%)	1.5	(41)
$Ca_v3.2$	8 (max 45%)	22	2.3		1 (9%)		(41)

Some compounds did not totally block the T-type current at the maximum dose tested. In such cases the values in the table represent the apparent IC_{50} with the maximum (max) block obtained in parentheses. Studies where only a single concentration was studied are noted by giving the concentration and the percent inhibition observed or NE if no significant effect was observed. The charge carrier used in each study is as follows (in mM): (reference 41) 2 Ca^{2+}, (50) 10 Ba^{2+}, (44) 1.8 Ca^{2+}, (91) 5 Ca^{2+}, (92) 51 Ba^{2+}, (93) 7 Ca^{2+}, (42) 3 Ca^{2+}, (94) 3 Ca^{2+}, (57) 10 Ca^{2+}, (90) 10 Ca^{2+}. Other abbreviations include: MPS, methylphenyl-succinimide; DRG, dorsal root ganglion; NB-1, neuroblastoma; nRT, reticular nucleus; and VB, ventrobasal.

systems, including the cloned channels (Table 2). With one notable exception (44), most studies have failed to confirm block of T-type currents at therapeutically relevant concentrations, leading to the suggestion that block of other ionic channels may be more relevant (45).

Some antiepileptics are characterized by hydantoin and imide molecular frameworks (Fig. 2). Hydantoins include phenytoin and phenobarbital and consist of a carbonyl-amine-carbonyl-amine moiety. Imides such as MPS and ethosuximide have the alternating carbonyl-amine functionality but without the additional carbonyl. It is reasonable to think that the T-channel protein would exhibit differential sensitivity to isomers of an antagonist. Such is the case with EMTBL, a cyclic thioester, where α-EMTBL exhibits blocking activity 5 times that of the β stereoisomer in rat dorsal root ganglion (DRG) (46). Chlordiazepoxide, a benzodiazepine tranquilizer that also has anti-convulsant effects, blocks both LVA and HVA channels with IC_{50}s of approximately 0.3 mM (47). Valproic acid is structurally the simplest of the antiepileptics listed in Fig. 2. Only partial block by valproic acid has been observed (Table 2). Zonisamide, a relatively new anti-epileptic, contains both a sulfonamide and oxazole functionality. It too was only capable of partial block. Based on a limited amount of experimental evidence, T-type channel block is thought to underlie the mechanism of action of both valproic acid and zonisamide.

Anesthetics

Although the precise mechanism of action of anesthetics is unknown, many studies have demonstrated that they are capable of blocking ion channels. Many general anesthetics appear to potentiate chloride currents through ligand-gated GABA and glycine channels. A newly described mechanism for inhalation anesthetics is that they hyperpolarize neurons by potentiating the activity of leak K^+ currents carried by the two pore domain channels such as TASK-1 (48). Table 3 lists the block of T-type calcium channels by various types of anesthetics, and their structures are shown in Fig. 3.

In contrast to the tissue variability noted above, there is general agreement between studies on the potency of various inhalation anesthetics (Table 3), although decreased sensitivity of cardiac T-type currents has been noted (49). Both isoflurane and halothane block T-type currents at therapeutically relevant concentrations of anesthetics (50). Block of high voltage-activated currents by halothane occurs at similar millimolar concentrations (51, 52).

Steroid analogs have been developed that are neuroactive, including some such as alphaxalone that are anesthetic (53). New compounds have been synthesized that do not interact with GABA or Na^+ channels, such as ACN and ECN (54). It is interesting to note that of the stereoisomers of ACN and ECN, it is the (+) enantiomer of both that was most active. This suggests that the alcohol and cyano functionalities are interchangeable, since the activities (+)-ECN and (+)ACN are essentially the same despite a 'swap' of the two functional groups. T-type currents of rat DRG neurons were partially blocked by (+)-ECN (40% maximal inhibition; IC_{50}, 0.3 μM), while (−)-ECN block occurred at 30-fold higher concentrations (54). It also produced partial block of HVA currents (approximately 30% maximal inhibition; IC_{50}, 9 μM). Among HVA currents, (+)-ACN appears to be somewhat selective for members of the Ca_v2 family (55).

Octanol (20 μM), an aliphatic alcohol, was reported to block LVA currents totally from inferior olivary neurons (56). Subsequent studies required much higher concentrations for block (Table 3). Studies on hippocampal CA1 pyramidal neurons indicated that octanol is non-selective,

Table 3. Anesthetics and barbiturates shown to inhibit T-type calcium currents

	Isoflurane	Enflurane	Halothane	Pentobarbital	Thiopental	Etomidate	Propofol	Ketamine	Octanol	Reference
	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(μM)	(mM)	(mM)	
DRG (neonatal rat)			0.1							(52)
DRG (rat)	0.3	1.2 (56%)	0.6	0.3		0.2	13	2.5	0.12	(41, 49, 50)
Hippocampus	1 (75%)								0.3 (53%)	(57, 95)
Ventricular myocytes	1.2 (20%)		1.4 (20%)							(49)
Adrenal glomerulosa	0.7 (47%)	1.2 (56%)	0.7 (24%)							(49)
GH3			1.3	1					0.24	(51, 90)
Thyroid carcinoma	0.7 (33%)	1.2 (46%)	0.7 (24%)							(49, 96)
$Ca_v3.1$	0.3			0.3	0.3	0.16	20	1.2	0.16	(41)
$Ca_v3.2$				0.3			27		0.22	(41)

The values represent either the IC_{50} determined from dose-response measurements or the percent inhibition (in parentheses) by a single concentration. The charge carrier used in each study is as follows (mM): (reference 52) 10 Ca^{2+}; (41) 2 Ca^{2+}; (49) adrenal glomerulosa: 20 Ca^{2+}, DRG: 2 Ca^{2+}, thyroid: 10 Ca^{2+}, ventricular myocyte: 2 Ca^{2+}; (50) 10 Ba^{2+}; (57) 2 Ca^{2+}; (95) 5 Ca^{2+}; (51), 10 Ca^{2+}; (90) 10 Ca^{2+}; (96) 10 Ca^{2+}.

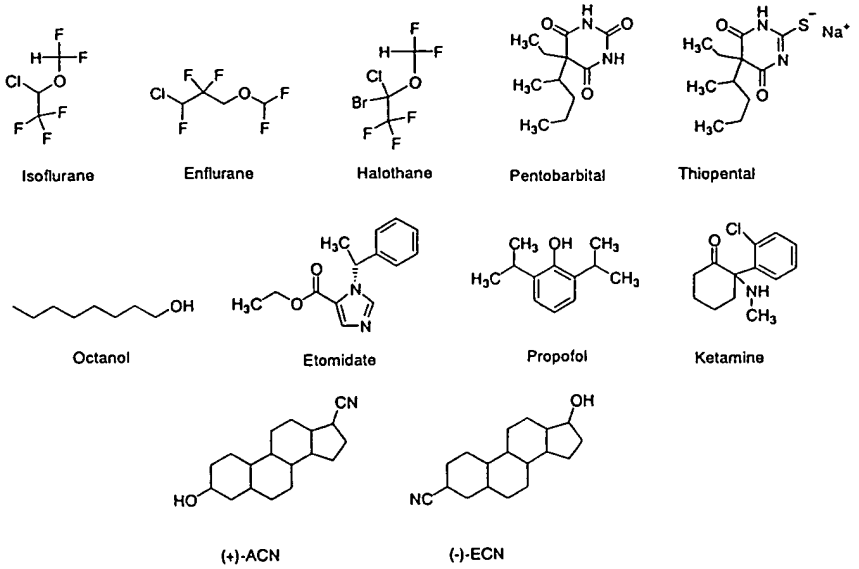

Fig. 3. Anesthetics shown to block T-type calcium current.

blocking T-, N- and L-type channels to a similar extent (57).

Antipsychotics

Antipsychotics, introduced in the 1950s, are used today in the treatment of psychotic disorders such as schizophrenia, the main phase of manic-depressive illness and other acute idiopathic psychotic illnesses (58). Anti-psychotics (neuroleptics) primarily act to block the D2 dopaminergic receptors, thereby inactivating dopamine neurotransmission in the forebrain. In addition, some neuroleptics can act on the D1 dopaminergic, 5-HT_2 serotinergic and α_2-adrenergic receptors (59).

The antipsychotics shown in Fig. 4 are characterized by varying degrees of aromaticity, and heterocyclic, non-aliphatic amines. The core amine heterocycles are all 1,4 di-substituted. Five of the six compounds have halogens (chlorine and fluorine) on the aromatic rings located primarily at the *para* position. With the exception of clozapine, all these compounds have short (2 – 3) carbon aliphatic chains connecting the heterocyclic amines to the aromatic rings. The most structurally similar compounds are penfluridol, fluspirilene and pimozide. The higher sensitivity of T-type channels to penfluridol might be attributed to a decrease in steric bulk and hydrogen bonding ability of the hydroxyl at the 4 position compared to the others. Despite strikingly different structures, thioridazine, clozapine and haloperidol have comparable activity in TT cells, but are still much less active than fluspiriline or penfluridol (60).

Of the three diphenylbutylpiperidines, penfluridol was the most effective at blocking T-type currents of human medullary thyroid cancer (TT) cells (60). In these cells, penfluridol blocked with an IC_{50} of 224 nM. It was also approximately tenfold more selective for LVA currents over HVA currents as 500 nM penfluridol inhibited 82% of the LVA current but only 20% of the HVA. Both penfluridol and pimozide block in a state-dependent manner, consistent with a lower affinity to the resting state (60, 61). Of all the compounds tested on T-type currents, these are the only class of drugs with IC_{50}'s in the sub-micromolar range (Table 4). Unfortunately, they are also very potent blockers of K^+ channels (26) and bind tightly to L-type channels (62).

Others

Amiloride is a potassium-sparing diuretic that acts by blocking a *non*-voltage-activated Na^+ channel in kidney epithelial cells (ENaC). It can also block voltage-gated channels, preferentially blocking LVA currents with little block of HVA channels or voltage-activated Na^+ channels (63). Studies using cardiac myocytes have confirmed the selectivity of amiloride for T- vs L-type channels (64, 65). Its selectivity for LVA currents has been a useful tool in a number of studies on neuronal Ca^{2+} currents (66). In contrast, most studies have not confirmed amiloride's sensitivity, requiring up to 30-fold higher concentrations (Table 5). Although this discrepancy might be explained by differential sensitivity of the T-type channel subtypes (67, 68), it does not explain discrepancies between studies on the same cell type. For example, using mouse spermato-

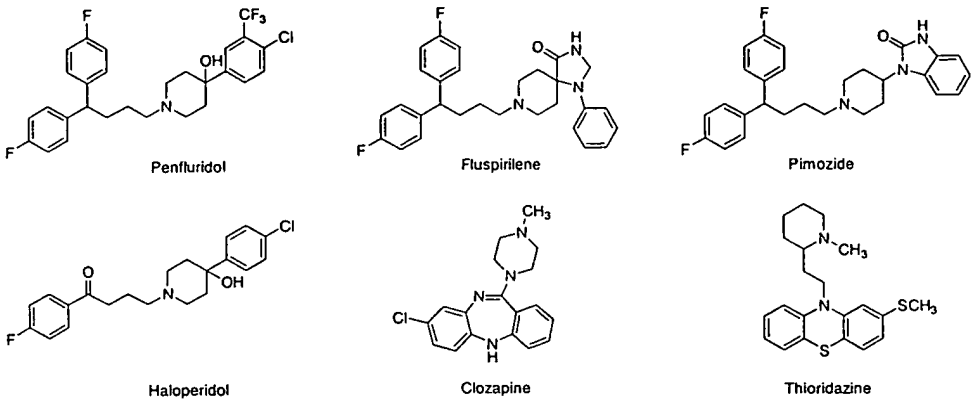

Fig. 4. Antipsychotics shown to inhibit T-type calcium channel activity.

Table 4. Antipsychotics shown to inhibit T-type calcium channel activity

	Penfluridol	Fluspiriline	Clozapine	Haloperidol	Thioridazine	Pimozide	Reference
Pituitary (rat)-GH4C1	1 (45%)	2 (64%)					(97)
Thyroid carcinoma (rat)	0.2						(60)
Thyroid carcinoma (human)	0.1 (70%)	1 (90%)	10	5 (31%)	10 (25%)		(60)
Spermatocytes (mouse)						0.5	(61)

All concentrations reported are micromolar and represent the IC_{50} values determined from dose-response measurements. Studies where only a single concentration was studied are noted by giving the concentration and the percent inhibition observed. The charge carrier used in each study is as follows (mM): (reference 97) 5 Ca^{2+}, (60) 10 Ca^{2+}, (61) 10 Ca^{2+}.

genic cells, Santi and co-workers (69) found that 0.5 mM amiloride only blocked 62% of the T-type current, while Arnoult et al. found that roughly half this concentration produced half-maximal block, reporting an IC_{50} of 0.24 mM (17). In addition to blocking ENaC channels, the usefulness of millimolar concentrations of amiloride is further limited by its block of transporters, such as the Na^+/H^+ and Na^+/Ca^{2+} transporters (70).

It has been hypothesized that blockade of T-type channels may provide a neuroprotective effect after ischemia (71, 72). To this end, a number of dimethylphenyl-piperazines (e.g., U-92032 (73)) and arylpiperadine analogs (e.g., SUN N5030 (72)) have been evaluated (Fig. 5). Although analogs have been described that are selective for LVA over HVA channels, they block voltage-gated Na^+ channels at similar concentrations. Similarly, the lamotrigine analog sipatrigine is a neuroprotective agent, is capable of blocking T-type channels, and can block Na^+ channels (74).

Tetranadrine, a plant alkaloid isolated from the root of *Stephania tetrandra*, was equally effective at blocking T-channels in adrenal glomerulosa cells, Y_1 adrenocortocal tumor cells and bullfrog cardiomyocytes at approximately 10 μM (Table 5). It also blocks L-type channels at similar concentrations (75).

In contrast to their potent effects on HVA channels, a selective peptide toxin for LVA channels has yet to be isolated from either spider or snail toxins. A scorpion toxin was isolated that appeared to be selective for LVA channels, since 350 nM could block currents through cloned T-type channels with no effect on the cloned HVA channels $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$ (76). Unfortunately its usefulness is limited by its ability to modify gating of Na^+ channels.

Conclusions

Because T-type calcium currents have been found in a variety of tissue and cell types, many physiological and pathophysiological functions for the channel have been proposed. T-type channels are most abundant in neurons, especially in medium-sized neurons from sensory ganglia (77). T-type currents have also been recorded from smooth muscle myocytes, pancreatic β cells, adrenocortical cells, osteoblasts, fibroblasts and glial cells (13). Physiological roles for the T-type channel include smooth muscle contraction, hormone (but not neurotransmitter) secretion and regulation of neuronal excitability, where they mediate rebound burst firing (13, 78). Increased T-type channel expression has been observed in proliferating and hypertrophic myocytes, leading to the hypothesis that they may also be involved in cell cycle progression (reviewed in

Table 5. Other compounds shown to block T-type calcium calcium channels

	Amiloride	U-92032	Tetrandrine	Reference
	mM	μM	μM	
DRG (chick)	0.03			(63)
DRG (rat)	0.076			(50)
Motoneuron	1 (74%)			(21)
Neuroblastoma, N18	0.03			(63)
Neuroblastoma, N1E-115		6 (50%)	2.5 (max 70%)	(73, 88)
Neuroblastoma, IMR-32	0.5 (58%)			(98)
Thalamus nRT	0.5 (41%)			(94)
Thalamus VB	0.5 (38%)			(94)
Hippocampus CA1	0.3 (43%)	0.5		(57, 99)
Atrial myocytes		1		(100)
Ventricular myocytes	0.23		10 (57%)	(64, 75)
Cardiac Purkinje	0.25 (50%)			(65)
GH3	1.55			(90)
Spermatocytes	0.24			(61)
$Ca_v3.2$	0.17			(67)

The charge carrier used in each study is as follows (in mM): (reference 63) 5 Ca^{2+}, (50) 10 Ba^{2+}, (21) 5 Ba^{2+}, (73) 50 Ba^{2+}, (88) 20 Ba^{2+}, (98) 10 Ca^{2+}, (94) 3 Ca^{2+}, (57) 10 Ca^{2+}, (99) 2 Ca^{2+}, (100) 5 Ca^{2+}, (64) 5.4 Ca^{2+}, (75) 1.8 Ca^{2+}, (65) 5 Ca^{2+}, (90) 10 Ca^{2+}, (61) 10 Ca^{2+}, (67) 15 Ba^{2+}. Abbreviations: DRG, dorsal root ganglion; NIE-115, mouse neuroblastoma; nRT, reticular nucleus; VB, ventrobasal.

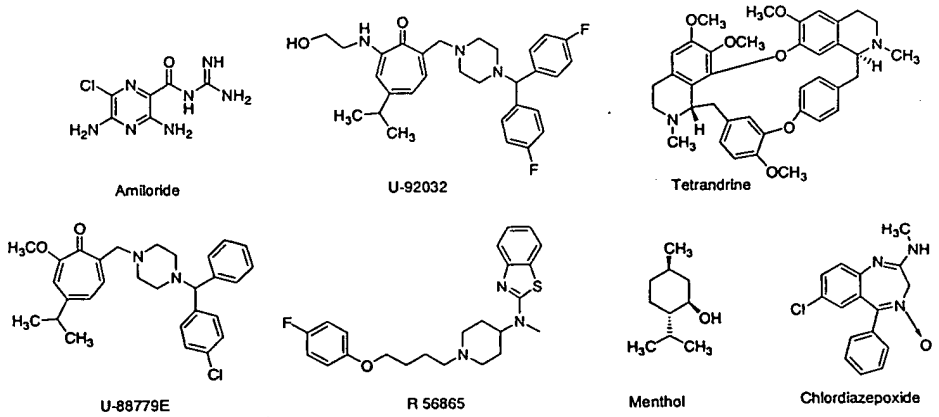

Fig. 5. A variety of structurally diverse compounds that block T-type calcium current.

ref. 13). Although to date there are no highly selective T-type channel blockers, studies using a variety of compounds that all have activity against these channels have led to the conclusion that selective blockers might be useful in regulating proliferation, blood pressure and abnormal neuronal firing. T-type channels may play a central role in thalamic dysrhythmias and therefore blockers may be useful not only against epilepsy but also a wide spectrum of neurological disorders (79). The clinical observation that high doses of anti-epileptics are analgesic, coupled with the observation that sensory neurons contain large T-type currents, leads to the speculation that a T-type channel drug may be useful in the treatment of neuropathic pain (80). It is important to note that in many neurons, T-type channels may serve as pacemaker currents, depolarizing the membrane to a level where Na^+ channels may fire. In these cases, there is a "pharmacological amplification" (81) of T-type channel inhibition such that even 10% block may lead to a pronounced effect on neuronal firing (82). It should also be noted that selectivity for T-type channels may be conferred by a drug's preference for inactivated channels. This may be particularly true in thalamic dysrhythmias since T-type channels are partially inactivated in states of wakefulness (83).

REFERENCES

1 Randall A and Tsien RW: Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons. J Neurosci **15**, 2995 – 3012 (1995)

2 Olivera BM, Miljanich GP, Ramachandran J and Adams ME: Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins. Annu Rev Biochem **63**, 823 – 867 (1994)

3 Ertel E, Campbell K, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E, Schwartz A, Snutch T, Tanabe T, Birnbaumer L, Tsien RW and Catterall WA: Nomenclature of voltage-gated calcium channels. Neuron **25**, 533 – 535 (2000)

4 Doyle DA, Cabral JM, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT and MacKinnon R: The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. Science **280**, 69 – 77 (1998)

5 Huber I, Wappl E, Herzog A, Mitterdorfer J, Glossmann H, Langer T and Striessnig J: Conserved Ca^{2+}-antagonist-binding properties and putative folding structure of a recombinant high-affinity dihydropyridine-binding domain. Biochem J **347 Pt 3**, 829 – 836 (2000)

6 Hofmann F, Lacinova L and Klugbauer N: Voltage-dependent calcium channels: from structure to function. Rev Physiol Biochem Pharmacol **139**, 33 – 87 (1999)

7 Klugbauer N, Dai S, Specht V, Lacinova L, Marais E, Bohn G and Hofmann F: A family of γ-like calcium channel subunits. FEBS Lett **470**, 189 – 197 (2000)

8 Wei X, Pan S, Lang W, Kim H, Schneider T, Perez-Reyes E and Birnbaumer L: Molecular determinants of cardiac Ca^{2+} channel pharmacology: subunit requirement for the high affinity and allosteric regulation of dihydropyridine binding. J Biol Chem **270**, 27106 – 27111 (1995)

9 Gee NS, Brown JP, Dissanayake VUK, Offord J, Thurlow R and Woodruff GN: The novel anticonvulsant drug, gabapentin (Neurontin) binds to the $\alpha_2\delta$ subunit of a calcium channel. J Biol Chem **271**, 5768 – 5776 (1996)

10 Abernethy DR and Schwartz JB: Drug therapy: calcium-antagonist drugs. N Engl J Med **341**, 1447 – 1457 (1999)

11 Triggle DJ: The pharmacology of ion channels: with particular reference to voltage-gated Ca^{2+} channels. Eur J Pharmacol **375**, 311 – 325 (1999)

12 Welling A, Ludwig A, Zimmer S, Klugbauer N, Flockerzi V and Hofmann F: Alternatively spliced IS6 segments of the alpha 1C gene determine the tissue-specific dihydropyridine sensitivity of cardiac and vascular smooth muscle L-type Ca^{2+} channels. Circ Res **81**, 526 – 532 (1997)

13 Ertel SI, Ertel EA and Clozel JP: T-type Ca^{2+} channels and pharmacological blockade: potential pathophysiological relevance. Cardiovasc Drugs Ther **11**, 723 – 739 (1997)

14 Mishra SK and Hermsmeyer K: Selective inhibition of T-type Ca^{2+} channels by Ro 40-5967. Circ Res **75**, 144 – 148 (1994)

15 Benardeau A and Ertel E: Selective block of myocardial T-type calcium channels by mibefradil: A comparison with the 1,4-dihydropyridine, amlodipine. *In* Selective Block of Myocardial T-type Calcium Channels by Mibefradil: A Comparison with the 1,4-Dihydropyridine, Amlodipine, Edited by Clozel JP, Nargeot J and Tsien RW, pp 386 – 394, Adis International Chester, UK (1997)

16 Kobrin I, Bieska G, Charlon V, Lindberg E and Pordy R: Anti-anginal and anti-ischemic effects of mibefradil, a new T-type calcium channel antagonist. Cardiology **89**, 23 – 32 (1998)

17 Arnoult C, Lemons JR and Florman HM: Voltage-dependent modulation of T-type calcium channels by protein tyrosine phosphorylation. EMBO J **16**, 1593 – 1599 (1997)

18 Martin RL, Lee JH, Cribbs LL, Perez-Reyes E and Hanck DA: Mibefradil block of cloned T-type calcium channels. J Pharmacol Exp Ther **295**, 302 – 308 (2000)

19 Monteil A, Chemin J, Leuranguer V, Altier C, Mennessier G, Bourinet E, Lory P and Nargeot J: Specific properties of T-type calcium channels generated by the human α1I subunit. J Biol Chem **275**, 16530 – 16535 (2000)

20 Perchenet L, Benardeau A and Ertel EA: Pharmacological properties of $Ca_v3.2$, a low voltage-activated Ca^{2+} channel cloned from human heart. Naunyn Schmiedebergs Arch Pharmacol **361**, 590 – 599 (2000)

21 Viana F, Van den Bosch L, Missiaen L, Vandenberghe W, Droogmans G, Nilius B and Robberecht W: Mibefradil (Ro 40-5967) blocks multiple types of voltage-gated calcium channels in cultured rat spinal motoneurones. Cell Calcium **22**, 299 – 311 (1997)

22 McDonough SI and Bean BP: Mibefradil inhibition of T-type calcium channels in cerebellar Purkinje neurons. Mol Pharmacol **54**, 1080 – 1087 (1998)

23 Bezprozvanny I and Tsien RW: Voltage-dependent blockade of diverse types of voltage-gated Ca^{2+} channels expressed in *Xenopus* oocytes by the Ca^{2+} channel antagonist mibefradil (Ro 40-5967). Mol Pharmacol **48**, 540 – 549 (1995)

24 Nilius B, Prenen J, Kamouchi M, Viana F, Voets T and Droogmans G: Inhibition by mibefradil, a novel calcium channel antagonist, of Ca^{2+}- and volume-activated Cl^- channels in macrovascular endothelial cells. Br J Pharmacol **121**, 547 – 555 (1997)

25 Liu JH, Bijlenga P, Occhiodoro T, Fischer-Lougheed J, Bader CR and Bernheim L: Mibefradil (Ro 40-5967) inhibits several Ca^{2+} and K^+ currents in human fusion-competent myoblasts. Br J Pharmacol **126**, 245 – 250 (1999)

26 Gomora JC and Enyeart JJ: Dual pharmacological properties of a cyclic AMP-sensitive potassium channel. J Pharmacol Exp Ther **290**, 266 – 275 (1999)

27 Gomora JC, Xu L, Enyeart JA and Enyeart JJ: Effect of mibefradil on voltage-dependent gating and kinetics of T-type Ca^{2+} channels in cortisol-secreting cells. J Pharmacol Exp Ther **292**, 96 – 103 (2000)

28 Eller P, Berjukov S, Wanner S, Huber I, Hering S, Knaus HG, Toth G, Kimball SD and Striessnig J: High affinity interaction of mibefradil with voltage-gated calcium and sodium channels. Br J Pharmacol **130**, 669 – 677 (2000)

29 Mehrke G, Zong XG, Flockerzi V and Hofmann F: The Ca^{++}-channel blocker Ro 40-5967 blocks differently T-type and L-type Ca^{++} channels. J Pharmacol Exp Ther **271**, 1483 – 1488 (1994)

30 Klugbauer N, Marais E, Lacinova L and Hofmann F: A T-type calcium channel from mouse brain. Pflugers Arch **437**, 710 – 715 (1999)

31 Cohen CJ, Spires S and Van Skiver D: Block of T-type Ca channels in guinea pig atrial cells by antiarrhythmic agents and Ca channel antagonists. J Gen Physiol **100**, 703 – 728 (1992)

32 Akaike N, Kostyuk PG and Osipchuk YV: Dihydropyridine-sensitive low-threshold calcium channels in isolated rat hypo-

thalamic neurones. J Physiol (Lond) **412**, 181 – 195 (1989)
33 Tarasenko AN, Kostyuk PG, Eremin AV and Isaev DS: Two types of low-voltage-activated Ca^{2+} channels in neurones of rat laterodorsal thalamic nucleus. J Physiol (Lond) **499**, 77 – 86 (1997)
34 Lievano A, Bolden A and Horn R: Calcium channels in excitable cells: divergent genotypic and phenotypic expression of alpha 1-subunits. Am J Physiol **267**, C411 – C424 (1994)
35 Kass RS: Voltage-dependent modulation of cardiac calcium channel current by optical isomers of Bay K 8644: implications for channel gating. Circ Res **61**, 11 – 15 (1987)
36 Romanin C, Seydl K, Glossmann H and Schindler H: The dihydropyridine niguldipine inhibits T-type Ca^{2+} currents in atrial myocytes. Pflugers Arch **420**, 410 – 412 (1992)
37 Bean BP: Nitrendipine block of cardiac calcium channels: high-affinity binding to the inactivated state. Proc Natl Acad Sci USA **81**, 6388 – 6392 (1984)
38 Richard S, Diochot S, Nargeot J, Baldy-Moulinier and Valmier J: Inhibition of T-type calcium currents by dihydropyridines in mouse embryonic dorsal root ganglion neurons. Neurosci Lett **132**, 229 – 234 (1991)
39 Kuga T, Sadoshima J, Tomoike H, Kanaide H, Akaike N and Nakamura M: Actions of Ca^{2+} antagonists on two types of Ca^{2+} channels in rat aorta smooth muscle cells in primary culture. Circ Res **67**, 469 – 480 (1990)
40 Twombly DA, Yoshi M and Narahashhi T: Mechanisms of calcium channel block by phenytoin. J Pharmacol Exp Ther **246**, 189 – 195 (1988)
41 Todorovic SM, Perez-Reyes E and Lingle CJ: Anticonvulsants but not general anesthetics have differential blocking effects on different T-type current variants. Mol Pharmacol **58**, 98 – 108 (2000)
42 Coulter DA, Huguenard JR and Prince DA: Characterization of ethosuximide reduction of low-threshold calcium current in thalamic neurons. Ann Neurol **25**, 582 – 593 (1989)
43 Coulter DA, Huguenard JR and Prince DA: Differential effects of petit mal anticonvulsants and convulsants on thalamic neurones: calcium current reduction. Br J Pharmacol **100**, 800 – 806 (1990)
44 Kostyuk PG, Molokanova EA, Pronchuk NF, Savchenko AN and Verkhratsky AN: Different action of ethosuximide on low- and high-threshold calcium currents in rat sensory neurons. Neuroscience **51**, 755 – 758 (1992)
45 Leresche N, Parri HR, Erdemli G, Guyon A, Turner JP, Williams SR, Asprodini E and Crunelli V: On the action of the anti-absence drug ethosuxamide in the rat and cat thalamus. J Neurosci **18**, 4842 – 4853 (1998)
46 Gross RA, Covey DF and Ferrendelli JA: Voltage-dependent calcium channels as targets for convulsant and anticonvulsant alkyl-substituted thiobutyrolactones. J Pharmacol Exp Ther **280**, 686 – 694 (1997)
47 Reuveny E, Twombly DA and Narahashi T: Chlordiazepoxide block of two types of calcium channels in neuroblastoma cells. J Pharmacol Exp Ther **264**, 22 – 28 (1993)
48 Sirois JE, Lei Q, Talley EM, Lynch C 3rd and Bayliss DA: The TASK-1 two-pore domain K^+ channel is a molecular substrate for neuronal effects of inhalation anesthetics. J Neurosci **20**, 6347 – 6354 (2000)
49 McDowell TS, Pancrazio JJ, Barrett PQ and Lynch C 3rd: Volatile anesthetic sensitivity of T-type calcium currents in various cell types. Anesth Analg **88**, 168 – 173 (1999)
50 Todorovic SM and Lingle CJ: Pharmacological properties of T-type Ca^{2+} current in adult rat sensory neurons: effects of anticonvulsant and anesthetic agents. J Neurophysiol **79**, 240 – 252 (1998)
51 Herrington J, Stern RC, Evers AS and Lingle CJ: Halothane inhibits two components of calcium current in clonal (GH3) pituitary cells. J Neurosci **11**, 2226 – 2240 (1991)
52 Takenoshita M and Steinbach JH: Halothane blocks low-voltage-activated calcium currents in rat sensory neurons. J Neurosci **11**, 1404 – 1412 (1991)
53 Gasior M, Carter RB and Witkin JM: Neuroactive steroids: potential therapeutic use in neurological and psychiatric disorders. Trends Pharmacol Sci **20**, 107 – 112 (1999)
54 Todorovic SM, Prakriya M, Nakashima YM, Nilsson KR, Han M, Zorumski CF, Covey DF and Lingle CJ: Enantioselective blockade of T-type Ca^{2+} current in adult rat sensory neurons by a steroid that lacks gamma-aminobutyric acid-modulatory activity. Mol Pharmacol **54**, 918 – 927 (1998)
55 Nakashima YM, Todorovic SM, Covey DF and Lingle CJ: The anesthetic steroid (+)-3α-hydroxy-5α-androstane-17β-carbonitrile blocks N-, Q-, and R-type, but not L- and P-type, high voltage-activated Ca^{2+} current in hippocampal and dorsal root ganglion neurons of the rat. Mol Pharmacol **54**, 559 – 568 (1998)
56 Llinas RR: The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function. Science **242**, 1654 – 1664 (1988)
57 Takahashi K, Wakamori M and Akaike N: Hippocampal CA1 pyramidal cells of rats have four voltage-dependent calcium conductances. Neurosci Lett **104**, 229 – 234 (1989)
58 Baldessarini RJ: Drugs and the treatment of psychiatric disorders. *In* Drugs and the Treatment of Psychiatric Disorders, Edited by Hardman JG, Limbird LE, Molinoff PB, Ruddon RW and Gilman AG, pp 399 – 430, McGraw-Hill, New York (1996)
59 Hardman JG, Limbird LE, Molinoff PB, Ruddon RW and Gilman AG: *In* The Pharmacological Basis of Therapeutics, pp 1 – 1905, McGraw-Hill, New York (1996)
60 Enyeart JJ, Biagi BA and Mlinar B: Preferential block of T-type calcium channels by neuroleptics in neural crest-derived rat and human C cell lines. Mol Pharmacol **42**, 364 – 372 (1992)
61 Arnoult C, Villaz M and Florman HM: Pharmacological properties of the T-type Ca^{2+} current of mouse spermatogenic cells. Mol Pharmacol **53**, 1104 – 1111 (1998)
62 King VF, Garcia ML, Shevell JL, Slaughter RS and Kaczorowski GJ: Substituted diphenylbutylpiperidines bind to a unique high affinity site on the L-type calcium channel. Evidence for a fourth site in the cardiac calcium entry blocker receptor complex. J Biol Chem **264**, 5633 – 5641 (1989)
63 Tang C-M, Presser F and Morad M: Amiloride selectively blocks the low threshold (T) calcium channel. Science **240**, 213 – 215 (1988)
64 Tytgat J, Vereecke J and Carmeliet E: Mechanism of cardiac T-type Ca channel blockade by amiloride. J Pharmacol Exp Ther **254**, 546 – 551 (1990)
65 Hirano Y, Fozzard HA and January CT: Characteristics of L- and T-type Ca^{2+} currents in canine cardiac Purkinje cells. Am J Physiol **256**, H1478 – H1492 (1989)
66 McCobb DP and Beam KG: Action potential waveform voltage-clamp commands reveal striking differences in calcium entry via low and high voltage-activated calcium channels. Neuron **7**,

119 – 127 (1991)
67 Williams ME, Washburn MS, Hans M, Urrutia A, Brust PF, Prodanovich P, Harpold MM and Stauderman KA: Structure and functional characterization of a novel human low-voltage activated calcium channel. J Neurochem **72**, 791 – 799 (1999)
68 Monteil A, Chemin J, Bourinet E, Mennessier G, Lory P and Nargeot J: Molecular and functional properties of the human α1G subunit that forms T-type calcium channels. J Biol Chem **275**, 6090 – 6100 (2000)
69 Santi CM, Darszon A and Hernandez-Cruz A: A dihydropyridine-sensitive T-type Ca^{2+} current is the main Ca^{2+} current carrier in mouse primary spermatocytes. Am J Physiol **271**, C1583 – C1593 (1996)
70 Kleyman TR and Cragoe EJ Jr.: Amiloride and its analogs as tools in the study of ion transport. J Membr Biol **105**, 1 – 21 (1988)
71 Im HK, Im WB and Tsuzuki K: Selective block of transient Ca channel current in mouse neuroblastoma cells by U-88779E. J Pharmacol Exp Ther **265**, 529 – 535 (1993)
72 Annoura H, Nakanishi K, Uesugi M, Fukunaga A, Miyajima A, Tamura-Horikawa Y and Tamura S: A novel class of Na^+ and Ca^{2+} channel dual blockers with highly potent anti-ischemic effects. Bioorg Med Chem Lett **9**, 2999 – 3002 (1999)
73 Ito C, Im WB, Takagi H, Takahashi M, Tsuzuki K, Liou SY and Kunihara M: U-92032, a T-type Ca^{2+} channel blocker and antioxidant, reduces neuronal ischemic injuries. Eur J Pharmacol **257**, 203 – 210 (1994)
74 McNaughton NC, Hainsworth AH, Green PJ and Randall AD: Inhibition of recombinant low-voltage-activated Ca^{2+} channels by the neuroprotective agent BW619C89 (Sipatrigine). Neuropharmacology **39**, 1247 – 1253 (2000)
75 Rubio LS, Garrido G, Llanes L and Alvarez JL: Effects of tetrandrine on Ca^{2+}- and Na^+-currents of single bullfrog cardiomyocytes. J Mol Cell Card **25**, 801 – 813 (1993)
76 Chuang RS, Jaffe H, Cribbs L, Perez-Reyes E and Swartz KJ: Inhibition of T-type voltage-gated calcium channels by a new scorpion toxin. Nat Neurosci **1**, 668 – 674 (1998)
77 Scroggs RS and Fox AP: Calcium current variation between acutely isolated adult rat dorsal root ganglion neurons of different size. J Physiol (Lond) **445**, 639 – 658 (1992)
78 Huguenard JR: Low threshold calcium currents in central nervous system neurons. Annu Rev Physiol **58**, 329 – 348 (1996)
79 Llinas RR, Ribary U, Jeanmonod D, Kronberg E and Mitra PP: Thalamocortical dysrhythmia: A neurological and neuropsychiatric syndrome characterized by magnetoencephalography. Proc Natl Acad Sci USA **96**, 15222 – 15227 (1999)
80 McQuay H, Carroll D, Jadad AR, Wiffen P and Moore A: Anticonvulsant drugs for management of pain: a systematic review. Br Med J **311**, 1047 – 1052 (1995)
81 Narahashi T: Neuroreceptors and ion channels as the basis for drug action: past, present, and future. J Pharmacol Exp Ther **294**, 1 – 26 (2000)
82 Huguenard JR and Prince DA: Intrathalamic rhythmicity studied in vitro: nominal T-current modulation causes robust antioscillatory effects. J Neurosci **14**, 5485 – 5502 (1994)
83 McCormick DA and Bal T: Sleep and arousal: thalamocortical mechanisms. Annu Rev Neurosci **20**, 185 – 215 (1997)
84 Kaneda M and Akaike N: The low-threshold Ca current in isolated amygdaloid neurons in the rat. Brain Res **497**, 187 – 190 (1989)
85 Takahashi K and Akaike N: Calcium antagonist effects on low-threshold (T-type) calcium current in rat isolated hippocampal CA1 pyramidal neurons. J Pharmacol Exp Ther **256**, 169 – 175 (1991)
86 Liu Y and Lasater EM: Calcium currents in turtle retinal ganglion cells. I. The properties of T- and L-type currents. J Neurophysiol **71**, 733 – 742 (1994)
87 Randall AD and Tsien RW: Contrasting biophysical and pharmacological properties of T-type and R-type calcium channels. Neuropharmacology **36**, 879 – 893 (1997)
88 Liu Y, Karpinski E, Rao MR and Pang PKT: Tetrandrine: a novel calcium channel antagonist inhibits type I calcium channels in neuroblastoma cells. Neuropharmacology **30**, 1325 – 1331 (1991)
89 Tytgat J, Vereecke J and Carmeliet E: Mechanism of L- and T-type Ca^{2+} channel blockade by flunarizine in ventricular myocytes of the guinea-pig. Eur J Pharmacol **296**, 189 – 197 (1996)
90 Herrington J and Lingle CJ: Kinetic and pharmacological properties of low voltage-activated Ca^{2+} current in rat clonal (GH3) pituitary cells. J Neurophysiol **68**, 213 – 232 (1992)
91 Kelly KM, Gross RA and Macdonald RL: Valproic acid selectively reduces the low-threshold (T) calcium current in rat nodose neurons. Neurosci Lett **116**, 233 – 238 (1990)
92 Kito M, Maehara M and Watanabe K: Mechanisms of T-type calcium channel blockade by zonisamide. Seizure **5**, 115 – 119 (1996)
93 Suzuki S, Kawakami K, Nishimura S, Watanabe Y, Yagi K, Seino M and Miyamoto K: Zonisamide blocks T-type calcium channel in cultured neurons of rat cerebral cortex. Epilepsy Res **12**, 21 – 27 (1992)
94 Huguenard JR and Prince DA: A novel T-type current underlies prolonged Ca^{2+}-dependent burst firing in GABAergic neurons of rat thalamic reticular nucleus. J Neurosci **12**, 3804 – 3817 (1992)
95 Study RE: Isoflurane inhibits multiple voltage-gated calcium currents in hippocampal pyramidal neurons. Anesthesiology **81**, 104 – 116 (1994)
96 McDowell TS, Pancrazio JJ and Lynch C 3rd: Volatile anesthetics reduce low-voltage-activated calcium currents in a thyroid C-cell line. Anesthesiology **85**, 1167 – 1175 (1996)
97 Enyeart JJ, Biagi BA, Day RN, Sheu SS and Maurer RA: Blockade of low and high threshold Ca^{2+} channels by diphenylbutylpiperidine antipsychotics linked to inhibition of prolactin gene expression. J Biol Chem **265**, 16373 – 16379 (1990)
98 Carbone E, Sher E and Clementi F: Ca currents in human neuroblastoma IMR32 cells: kinetics, permeability, and pharmacology. Pflugers Arch **416**, 170 – 179 (1990)
99 Avery RB and Johnston D: Ca^{2+} channel antagonist U-92032 inhibits both T-type Ca^{2+} channels and Na^+ channels in hippocampal CA1 pyramidal neurons. J Neurophysiol **77**, 1023 – 1028 (1997)
100 Xu X and Lee KS: A selective blocker for rested T-type Ca^{++} channels in guinea pig atrial cells. J Pharmacol Exp Ther **268**, 1135 – 1142 (1994)

Voltage-Gated Calcium Channels

SIGMA-ALDRICH

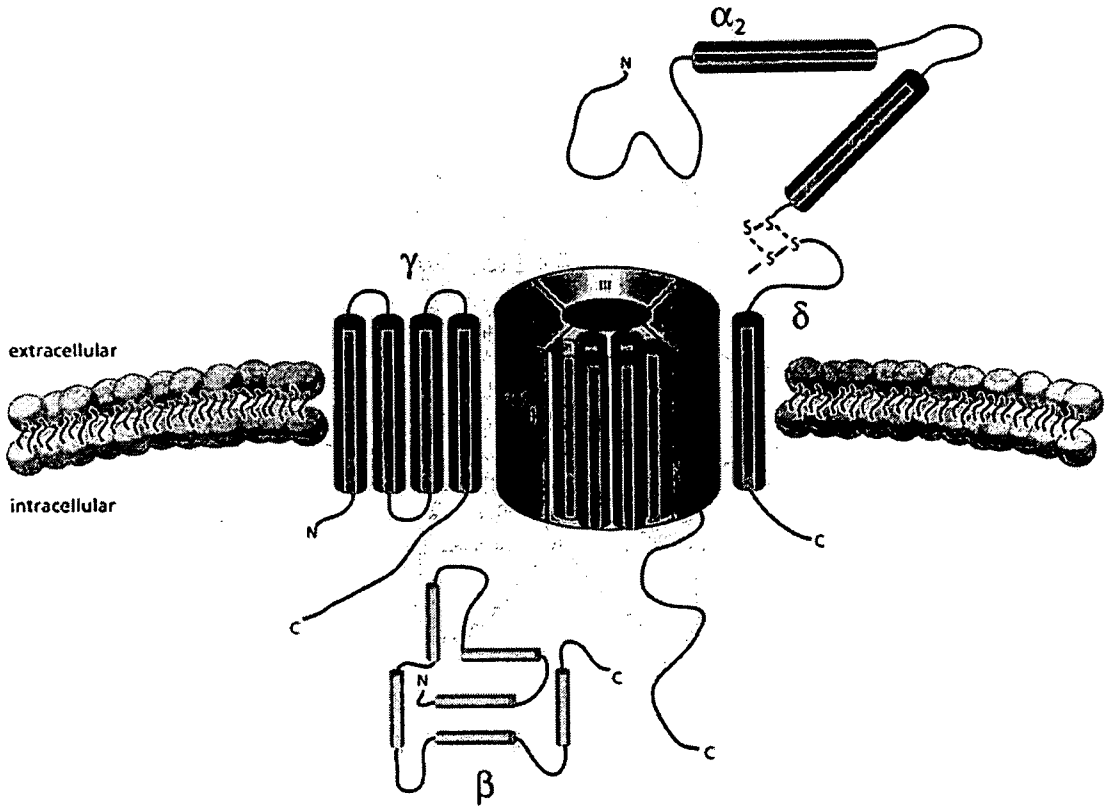

Exhibit D page 1

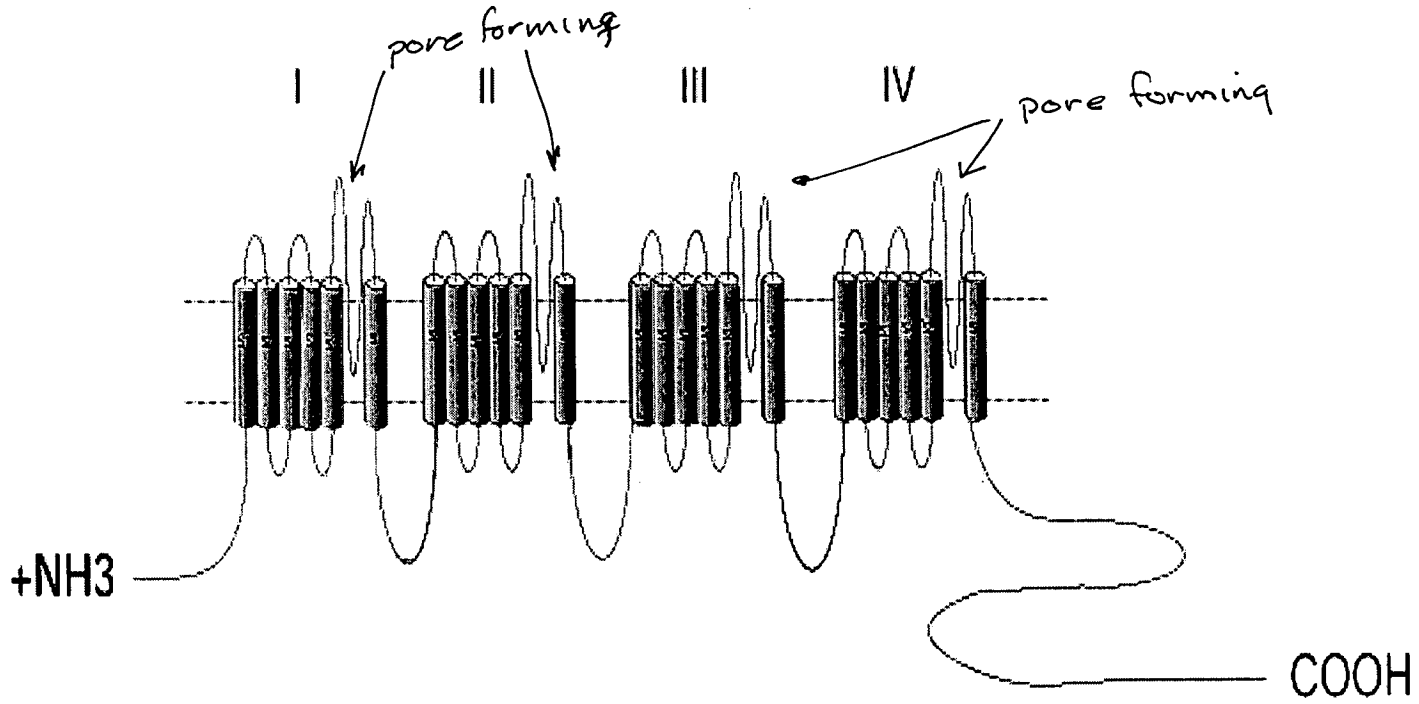

Exhibit D page 2